Report of the visit to the laboratory of Dr. Francisco Ciruela at the University of Barcelona, Spain

During my research stay in Barcelona, I developed a program of activities within the research project entitled "Molecular interaction between D_1 dopamine and Corticotrophin releasing hormone type-2 receptors (CRH-R_2)".

Introduction

G protein-coupled receptors (GPCRs) possess unique structural characteristics and a subcellular localization that make them able to interact with a large array of proteins at the plasma membrane level. Interestingly, within the proteins described to interact with GPCRs include their own family members. Classically, these receptors have been considered as independent functional units so the discovery of homo- and heteromeric GPCRs has revolutionized the way of tackling the study of their functionality. Spatiotemporal characterization of protein–protein interactions is essential in determining the molecular mechanisms of intracellular signaling processes. Although convincing, the immunoprecipitation studies to determine the interaction between proteins require the solubilization of membrane receptor, which does not exclude that the dimers observed may be artifacts of to the solubilization process with detergents, due to the hydrophobic nature of membrane receptors. A direct demonstration that these complexes exist in living cells has been possible thanks to the development and use of biophysical methods based on the resonance energy transfer (RET), for instance fluorescence–RET (FRET) and bioluminescence-RET (BRET).

The functional and/or molecular interactions between GPCRs had provided interesting findings on the regulation of glutamate release in brain nuclei. In the striatum, Rodrigues et al. (2005) by using immunofluorescence in synaptosomes showed the co-localization of adenosine A_{2A} receptors ($A_{2A}R$) and metabotropic glutamate type 5 receptors ($mGlu_5R$) in glutamatergic terminals in the striatum. Thus, by means of a release assay performed in striatal synaptosomes they clearly demonstrated that the simultaneous activation of the $A_{2A}R$ and $mGlu_5R$ produced a synergistic facilitation of the glutamate release. Interestingly, such interaction would modulate the neuroprotection processes within the striatum. In addition, Ciruela et al. (2006) showed that glutamatergic transmission in the striatum is modulated by the interaction between adenosine receptors. In brief, by means of immunohistochemistry and co-immunoprecipitation experiments performed in rat striatal synaptosomes, they showed that the adenosine A_1 receptor (A_1R) and the $A_{2A}R$ form heteromers which are located in the glutamatergic terminals of the striatum. Interestingly, when the glutamate release was analyzed they demonstrated that these heteromers are responsible of the adenosine-mediated modulation of the striatal glutamatergic transmission. In addition, it has been shown recently a Corticotrophin Releasing Hormone (CRH)-mediated sensitization of glutamate release in the ventral tegmental area (VTA) of cocaine-experienced rats due to the activation of VTA CRH receptor type-2 (CRH-R_2) (Wang et al 2005, 2007). Also, the activation of dopamine receptors type-1 (D_1) induces VTA glutamate release in cocaine treated rats (Kalivas and Duffy 1998).

The principal aim of my PhD thesis is centered on the study of the molecular and cellular mechanisms responsible for the sensitization of glutamate release in VTA mediated by the repeated administration of cocaine.
The hypothesis of my PhD thesis is that the ***repeated administration of cocaine induces the interaction between dopamine D_1 and CRH-R_2 receptors in glutamatergic terminals of VTA, and that this interaction determines the increase in extracellular glutamate levels observed following stressful stimuli in rats exposed to cocaine***.

Materials and methods

Accordingly, to demonstrate this hypothesis we propose three specific aims one of which consists in showing the molecular interaction between dopamine D_1 and CRH-R_2 receptors. Thus, we developed a program of activities in the laboratory of Dr. Ciruela in Barcelona to achieve this aim, briefly:

1) To determine the interaction between dopamine D_1 and CRH-R_2 receptors by means of BRET experiments.

2) To determine the interaction between dopamine D_1 and CRH-R_2 receptors by means of FRET-FRAP experiments.

The following receptor constructs were used:

- CRH-R_2-*R*luc, human CRH-R_2 receptor fused to *Renilla* luciferase.
- D_1-YFP, human D_1 dopamine receptor fused to yellow fluorescent protein.
- D_1-*R*luc human D_1 dopamine receptor fused to *Renilla l*uciferase.
- CRH-R_2-YFP, human CRH-R_2 receptor fused to yellow fluorescent protein.
- CRH-R_2-CFP, human CRH-R_2 receptor fused to cyan fluorescent protein.

Through the program developed in Barcelona I learned:

- To work in HEK293 cells expressing:
    i. CRH-R_2-Rluc and D_1-YFP receptors to measure the potential interaction between both receptors using BRET.
    ii. D_1-Rluc and CRH-R_2-YFP receptors to measure the potential interaction between both receptors using BRET.
- To work in HEK293 cells expressing CRH-R_2-CFP and D_1-YFP receptors to measure the potential interaction between both receptors using FRET-FRAP (FRAP:fluorescence recovery after acceptor photobleach).

Cell Cultures
For all experiments HEK-293T cells were used. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM L-glutamine, 100 UI/ml penicillin/streptomycin, and 5% (v/v) heat-inactivated fetalbovine serum (FBS) (all supplements were from Invitrogen). Cells were maintained at 37ºC in an atmosphere of 5% CO2, and were passaged when they were 80–90% confluent, twice a week.

Transfections

To demonstrate heteromerization of dopamine D_1 and CRH-R_2 receptors, we performed BRET experiments using D_1-Rluc and CRH-R_2-YFP constructs or CRH-R_2-Rluc and D_1-YFP contructs and FRET experiments using D_1-YFP and CRH-R_2-CFP constructs. All constructs had the coding sequence of *Rluc* or the fluorescent protein cloned on the c-terminal end of each receptor. For BRET experiments HEK-293T cells were transfected with 1 μg of DNA for the construct CRH-R_2-Rluc or D1-Rluc with variable amounts of DNA for the construct of D_1-YFP or CRH-R2-YFP, respectively. For other hand, for FRET-FRAP experiments, HEK-293T cells were transfected with 2 μg of DNA for the construct CRH-R_2-CFP and 2 μg of D_1-YFP. FRET and BRET experiments were performed 48 h after transfection.

BRET Experiments

Cells were rapidly washed twice in HBSS with 10mM glucose, detached, and resuspended in the same buffer containing 1mM EDTA. To control the cell number, sample protein concentration was determined using a BCA kit (Pierce) using BSA dilutions as standards. To quantify fluorescence of CRH-R2-EYFP, cells (20 mg protein) were distributed in duplicated 96-well microplates (black plates with a transparent bottom) and read in a FLUOstar (BMG LABTECH). YFP fluorescence was the fluorescence of the sample minus the fluorescence of cells not expressing CRH-R2-EYFP. For BRET measurement, the equivalent of 20 mg of cell suspension were distributed in triplicates in 96-well microplates white plates with white bottom and 5 mM coelenterazine H was added. After 1 min, the readings were collected using a FLUOstar that allows the integration of the signals detected in the filter at 485 nm (440–500 nm) and the 530nm (510–590 nm). To quantify luminescence of Rluc, readings were taken after 10 min of adding 10 μl coelenterazine H. The BRET signal was determined by calculating the ratio of the light emitted by EYFP (510–590 nm) over the light emitted by the Rluc (440–500 nm). The net BRET values were obtained by subtracting the BRET background signal detected when Rluc-tagged construct was expressed alone. Curves were fitted using a nonlinear regression and one-phase exponential association fit equation (One site binding (hyperbola) Y=Bmax*X/(Kd+X)).

FRET-FRAP Experiments

Cells were growth in polylysine and 48 h after transfection we made FRET-FRAP experiments. In these experiments, single cells were excited with light from a polychrome IV (TILL Photonics) and the illumination time was typically set to 25 ms applied with a frequency of 40 Hz. All recordings were performed in real-time, while cells were continuously superfused with HBSS pH 7,4. Detected FRET signals were digitalized with an AD converter (Digidata1322A, Axon Instruments, Union City, CA) and stored on a personal computer by using CLAMPEX 9.0 software (Axon Instruments). After this initial recording the photodestruction or photobleaching of the YFP fluorophore was then achieved by continuous irradiation at 510 nm for 10 min. Finally, a postbleching recording as described above was performed. The eFRET media was calculated with GraphPad Prism software.

Results

To demonstrate a direct physical interaction between dopamine D1 and CRH-R2 receptors, BRET was carried out in living HEK293 cells transfected with cDNAs encoding the fusion proteins CRH-R2-Rluc and D1-YFP. After transfection, the receptors expression was high at the membrane level (Figure 1). As energy transfer between two specifically interacting proteins has to reach a plateau, a saturable BRET curve was obtained for the CRH-R2-Rluc/D1-YFP pair when constant amounts of the cDNA for the Rluc construct were co-transfeted with increasing amounts of the plasmid cDNA for the YFP construct (Figure 1). Maximum net BRET was 139.50 and BRET50 was 21.25 attained at a relatively low D1-YFP/CRH-R2-Rluc ratio. As negative controls, no significant BRET was obtained in a mixture of cells transfected with CRH-R2-Rluc with GABAb2-YFP observed as the linear curve (Figure 1). These results indicate that the BRET signal obtained using CRH-R2-Rluc/D1-YFP was specifically due to CRH-R2–D1 receptor heteromerization.

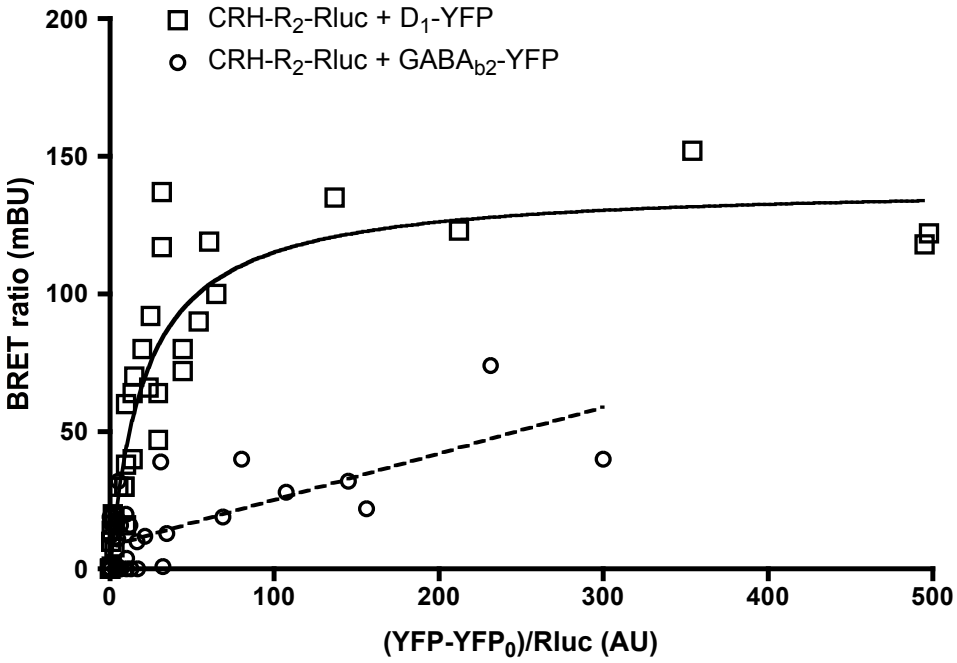

Figure 1. Interaction between CRH-R_2-Rluc and D_1-YFP receptors

To confirm the interaction between these receptors, we also performed BRET experiments were carried out in living HEK293 cells transfected with cDNAs encoding the fusion proteins D1-Rluc and CRH-R2-YFP. After transfection, the receptors expression was high at the membrane level (Figure 2). As energy transfer between two specifically interacting proteins has to reach a plateau, a saturable BRET curve was obtained for the D1-Rluc/ CRH-R2-YFP pair when constant amounts of the cDNA for the Rluc construct were co-transfeted with increasing amounts of the plasmid cDNA for the YFP construct (Figure 2). Maximum net BRET was 148.50 and BRET50 was 77.12 attained at a relatively low CRH-R2-YFP/D1-Rluc ratio. As negative controls, no significant BRET was obtained in a mixture of cells transfected with D1-Rluc with GABAb2-YFP (Figure 2). These results indicate that the BRET signal obtained using D1-Rluc/ CRH-R2-YFP was specifically due to CRH-R2–D1 receptor heteromerization.

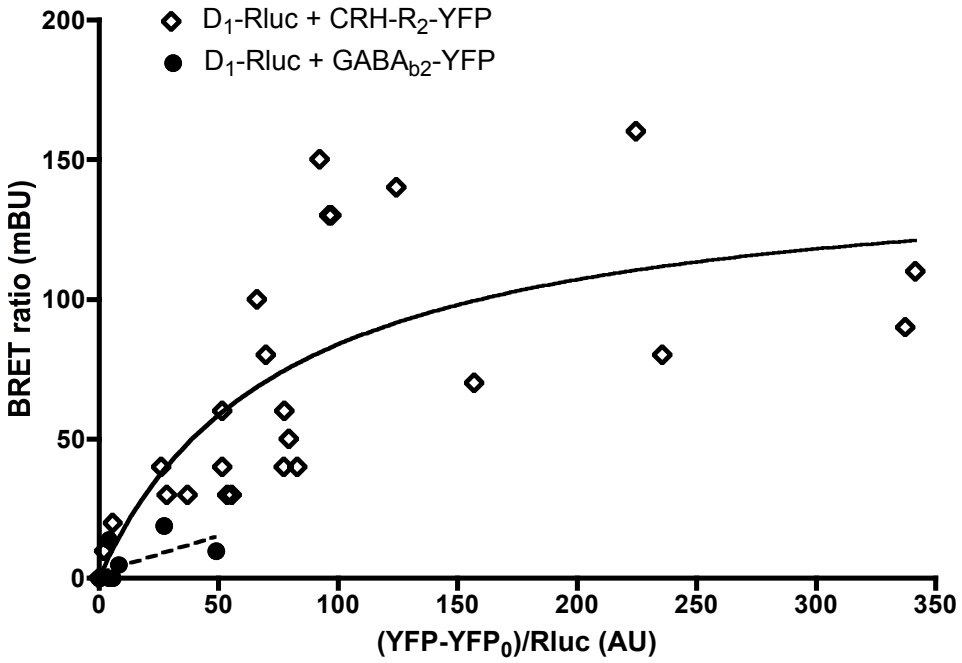

Figure 2. Interaction between D_1-Rluc and CRH-R_2-YFP receptors.

Moreover, to corroborate this interaction, we use cyan (CFP) and yellow (YFP) variants of the green fluorescent protein (GFP) can be used for FRET-FRAP in live cells. FRET was carried out in living HEK293 cells transfected with cDNAs encoding the fusion proteins CRH-R2-CFP and D1-YFP. When CRH-R2-CFP is excited and FRET occurs, CRH-R_2-CFP emission decreases and D_1-YFP emission increases (Figure 3). The presence of specific receptor heterocomplexes in transfected cells can therefore be detected by FRET measurements between two receptors. For donor can measure specific CRH-R2-D1 interactions, the efficiency of energy transfer between CRH-R2-CFP and D1-YFP FRAP. After performing the photobleaching for 10 minutes, the results show that the intensity of D1-YFP decreased by 60%. Increasing the intensity of CRH-R2-CFP obtained FRET efficiency (eFRET) of 11%. All these data have shown a physical or molecular interaction between dopamine D1 and CRH-R2 receptors.

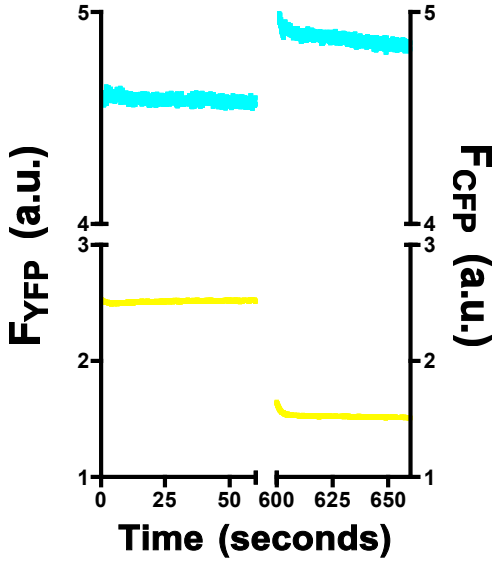

Figure 3. Interaction between CRH-R_2-CFP and D_1-YFP receptors

Conclusion

This research stay at the laboratory of Dr. Francisco Ciruela allowed me the acquisition of new techniques that I expect to employ in my future research. It is undoubtedly that this experience gave me the expertise to complete a more successful work. I am convinced that considerable value is added to a higher education if you can take part of these kinds of International Events that facilitate the exchange of scientific information relevant to molecular biology between investigators from other countries.

References

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